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(57) Abstract

Genetic markers in the porcine melanocortin-4 receptor (MC4R) gene are disclosed which are associated with fat content, growth rate, and feed consumption. Further, novel sequence data from regions of the gene are disclosed which may be used in a PCR test to screen for the presence of the marker. The genetic marker may be used to screen animals for breeding purposes which have the desired traits regarding fat content, growth rate, and feed consumption. Kits which take advantage of the PCR test are also disclosed.

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TITLE: MELANOCORTIN-4 RECEPTOR GENE AND USE AS A GENETIC MARKER FOR FAT CONTENT, WEIGHT GAIN, AND/OR FEED CONSUMPTION OF ANIMALS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application Serial No. 60/094,287 filed July 27, 1998 and U.S. Provisional Application Serial No. 60/116,186, filed January 15, 1999, the disclosures of which are hereby incorporated by reference.

GRANT REFERENCE CLAUSE

This invention was supported at least in part by grants from the United States Department of Agriculture through the Iowa Agriculture and Home Economics Experiment Station (IaHees) and Project Number IOW03148 (Hatch Funds). The United States government may have certain rights in this invention.

FIELD OF THE INVENTION

The present invention relates to a method of genetically evaluating animals by assaying for the presence of at least one genetic marker which is indicative of one or more of the traits of fat content, growth rate, and feed consumption. In particular, the method analyzes for variation in the melanocortin-4 receptor (MC4R) gene which is indicative of these traits. Even more particularly, the method analyzes for a polymorphism in the MC4R gene.

BACKGROUND OF THE INVENTION

There is an increasing consumer demand for meat products having low fat content. This demand is fueled by accumulating evidence in the scientific literature that a high consumption of animal fat, especially fat with a high proportion of saturated fatty acids, represents a significant health hazard, including risk for cardiovascular disease. Other health concerns associated with high fat meats include their high content of cholesterol and the addition of relatively high amounts of salt which are added to improve the binding characteristics since salt aids in extracting the native

water binding component myosin from the meat. Furthermore, an increasing number of consumers find meat products containing chemical additives such as phosphates, emulsifying additives, and anti-oxidants less acceptable.

Faced with consumers who seek a healthier meat product, meat producers are continually pressed to offer cheaper and healthier products.

Cheaper products, of course, come from lowering costs of production. Producers are always interested in improving the growth rate and feed conversion of their animals. Lower production costs come from the shorter time to market and lower costs of feeding an animal. This can increase the profit margin in the livestock industry and/or result in lower prices to the consumer.

By being able to select for animals which have the aforementioned traits, producers can raise animals with these desirable characteristics. Selection for desirable traits has traditionally been done using breeding techniques.

Genetic differences exist among individual meat producing animals as well as among breeds which can be exploited by breeding techniques to achieve animals with these desirable characteristics. For example, Chinese breeds are known for reaching puberty at an early age and for their large litter size, while American breeds are known for their greater growth rates and leanness. Thus, it would be desirable to combine the best characteristics of both types of these breeds, thereby improving pork production.

Often, however, heritability for desired traits is low, for example, heritability for litter size is around 10%-15%. Standard breeding methods which select individuals based upon phenotypic variations do not take fully into account genetic variability or complex gene interactions which exist. Therefore, there is a need for an approach that deals with selection for leanness, growth rate, and feed consumption at the cellular or DNA level. This method will provide a method for genetically evaluating animals to enable breeders to more accurately select those animals which not only phenotypically express desirable traits but those which express favorable underlying genetic criteria. This has largely been accomplished to date by marker assisted selection.

Restriction fragment length polymorphism (RFLP) analysis has been used by several groups to study pig DNA. Jung et al., Theor. Appl. Genet., 77:271-274 (1989), incorporated herein by reference, discloses the use of RFLP techniques to show genetic

variability between two pig breeds. Polymorphism was demonstrated for swine leukocyte antigen (SLA) Class I genes in these breeds. Hoganson et al., Abstract for Annual Meeting of Midwestern Section of the American Society of Animal Science, March 26-28, 1990, incorporated herein by reference, reports on the polymorphism of swine major histocompatibility complex (MHC) genes for Chinese pigs, also demonstrated by RFLP analysis. Jung et al., Animal Genetics, 26:79-91 (1989), incorporated herein by reference, reports on RFLP analysis of SLA Class I genes in certain boars. The authors state that the results suggest that there may be an association between swine SLA/MHC Class I genes and production and performance traits. They further state that the use of SLA Class I restriction fragments, as genetic markers, may have potential in the future for improving pig growth performance.

The ability to follow a specific favorable genetic allele involves a novel and lengthy process of the identification of a DNA molecular marker for a major effect gene. The marker may be linked to a single gene with a major effect or linked to a number of genes with additive effects. DNA markers have several advantages; segregation is easy to measure and is unambiguous, and DNA markers are co-dominant, i.e., heterozygous and homozygous animals can be distinctively identified. Once a marker system is established selection decisions could be made very easily, since DNA markers can be assayed any time after a tissue or blood sample can be collected from the individual infant animal.

The use of genetic differences in receptor genes has become a valuable marker system for selection. For example, United States Patents 5,550,024 and 5,374,526 issued to Rothschild et al. disclose a polymorphism in the pig estrogen receptor gene which is associated with larger litter size, the disclosure of which is incorporated herein by reference. United States application serial number 08/812,208 discloses polymorphic markers in the pig prolactin receptor gene which are associated with larger litter size and overall reproductive efficiency.

It can be seen from the foregoing that a need exists for a method for selecting animals with the improved metabolic traits regarding fat content, growth rate, and feed consumption.

SUMMARY OF THE INVENTION

An object of the present invention is to provide a genetic marker based on or within the MC4R gene which is indicative of fat content, growth rate, and/or feed consumption.

Another object of the invention is to provide an assay for determining the presence of this genetic marker.

A further object of the invention is to provide a method of evaluating animals that increases accuracy of selection and breeding methods for the desired traits.

Yet another object of the invention is to provide a PCR amplification test which will greatly expedite the determination of presence of the marker.

An additional object of the invention is to provide a kit for evaluating a sample of animal DNA for the identified genetic marker.

These and other objects, features, and advantages will become apparent after review of the following description and claims of the invention which follow.

This invention relates to the discovery of a polymorphism within the melanocortin-4 receptor (MC4R) gene which is associated with fat content, growth rate, and feed conversion traits in animals. According to the invention, the association of the MC4R polymorphism with the trait(s) enables genetic markers to be identified for specific breeds or genetic lines. The *TaqI* restriction pattern which identifies the polymorphism is used to assay for the presence or absence of markers associated with the desirable metabolic traits. The breed-dependent marker genotype (i.e., a marker in some breeds and a nonmarker in others) consists of a polymorphism within MC4R, a guanine to adenine transition at position 678 of the PCR product (a missense mutation of aspartic acid codon (GAU) into asparagine codon (AAU) at position 298 amino acid of the MC4R protein). The invention includes assays for detection of the marker as well as the sequence characterization of the polymorphism and includes novel sequences in the MC4R gene which may be used to design amplification primers for such an assay. Additionally, the invention includes a method for using the assay in breeding programs for animal selection and a kit for performing the assay.

Definitions

As used herein, "low fat content" or "leanness" means a biologically significant decrease in body fat relative to the mean of a given population.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is the sequence listing for MC4R in pigs (SEQ ID NO:1). "X" represents the site of the polymorphism.

Figure 2 represents a comparison of the DNA sequence between the human (SEQ ID NO:2) and the porcine (SEQ ID NO:3) MC4R gene.

Figure 3 represents a comparison of the amino acid sequence between the human (SEQ ID NO:4) and the porcine (SEQ ID NO:5) MC4R gene.

Figures 4a, 4b, and 4c are linkage reports for MC4R from CRI-MAP.

Figure 5 depicts partial nucleotide and amino acid sequences (SEQ ID NO:12) of the porcine MC4R gene. The amino acid translation shows an amino acid substitution at codon 298.

Figure 6 is an electrophoresis gel of *TaqI* digestion of the PCR product. Molecular marker (M) and MC4R genotypes are indicated at the top of each lane.

Figure 7 depicts multiple-alignments of the putative seventh transmembrane domain of porcine MC4R with other MCRs and GPCRs. The "*" represents the predicted sequence positions for porcine MC4R. The other amino acid sequences were obtained from the GenBank database (accession numbers P32245, P70596, P41983, P56451, P34974, P41968, P33033, Q01718, Q01726, Q28031, AF011466, P21554, P18089, P30680, P47211). The missense variant in porcine MC4R substituted amino acid N for D in the position marked with an arrow. The Asp (D) residue is highly conserved among MCRs, and the Asn (N) residue is well conserved in most other GPCRs.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

Obesity is a disease affecting energy balance. The control of energy metabolism is simple: store excessive energy as fat and manage the energy to avoid superfluous energy storage, i.e., obesity. Although several genes and signaling systems have been implicated in obesity, there has been little known about the interconnection of energy homeostatic mechanism and genetic polymorphism. The melanocortin-4 receptor (MC4R) has been shown to be an important mediator of long term weight homeostasis. MC4R antagonists can increase food intake and body weight during chronic administration. Skuladottir, G.V.,

et al., "Long term orexigenic effect of a novel melanocortin 4 receptor selective antagonist", British J. of Pharm., 126(1):27-34 (1999).

Lu et al. (1994) suggested that the melanocortin receptors are involved in controlling food intake and energy balance through studying its antagonism to the *agouti* obesity syndrome. Huszar et al. (1997) found that inactivation of the melanocortin-4 receptor gene (MC4R) resulted in a maturity onset obesity syndrome in mice and demonstrated a major role of MC4R protein in the regulation of energy balance related to the *agouti* obesity syndrome. In addition, the MC4R protein mediates the effects of leptin, one of the important signaling molecules in energy homeostasis (Seeley et al. 1997).

According to the present invention, a variant or polymorphism in the MC4R gene has been located, and this genetic variability is associated with phenotypic differences in the metabolic traits of fat content, growth rate, and/or feed consumption.

In one embodiment of the invention, an assay is provided for detection of presence of a desirable genotype. The assay involves amplifying the genomic DNA purified from blood, tissue, semen, or other convenient source of genetic material by the use of primers and standard techniques, such as the polymerase chain reaction (PCR), then digesting the DNA with a restriction enzyme (e.g., *Taq I*) so as to yield gene fragments of varying lengths, and separating at least some of the fragments from others (e.g., using electrophoresis).

The fragments may also be detected by hybridizing with a nucleotide probe (e.g., radio-labeled cDNA probes) that contains all or at least a portion of the MC4R gene cDNA sequence to the separated fragments and comparing the results of the hybridization with assay results for a gene sequence known to have the marker or a sequence known to not have the marker. Selection and use of probes for detection of MC4R sequences based on the known and disclosed MC4R sequences is generally known to those skilled in the art. The probe may be any sequence which will hybridize to the separated digestion products and allow for detection.

Another embodiment of the invention provides a kit for assaying the presence in a MC4R gene sequence of a genetic marker. The marker being indicative of inheritable traits of fat content, growth rate, and/or feed consumption. The kit in a preferred embodiment also includes novel PCR primers comprising 4-30 contiguous bases on either side of the

polymorphism to provide an amplification system allowing for detection of the *Taq I* polymorphism by PCR and *Taq I* digestion of PCR products. The preferred primers are SEQ ID NO:8 and SEQ ID NO:9.

A further embodiment comprises a breeding method whereby an assay of the above type is conducted on a plurality of gene sequences from different animals or animal embryos to be selected from and based on the results, certain animals are either selected or dropped out of the breeding program.

According to the invention, the polymorphism in the MC4R gene, identifiable by the *Taq I* restriction pattern, is disclosed. As is known in the art, restriction patterns are not exact determinants of the size of fragments and are only approximate. The polymorphism is identifiable by three bands from a *Taq I* digestion of the PCR product, 466, 225, and 76 base pairs (bp) for one homozygous genotype (allele 1); two bands, 542 and 225 bp for another homozygous genotype (allele 2); and four bands for the heterozygous genotype (542, 466, 225, and 76 bp). The marker for leanness and lower feed intake is identifiable by the 466/225/76 bands, except for the Chinese pigs, where the Chinese pigs' marker for leanness is the 542/225 bands. The marker for faster rate of gain is identifiable by the 542/225 bands.

In addition, the polymorphism associated with the pattern has been identified at the nucleotide level. The polymorphic *Taq I* site was sequenced along with the general surrounding area. See SEQ ID NO: 1. The sequences surrounding the polymorphism have facilitated the development of a PCR test in which a primer of about 4-30 contiguous bases taken from the sequence immediately adjacent to the polymorphism is used in connection with a polymerase chain reaction to greatly amplify the region before treatment with the *Taq I* restriction enzyme. The primers need not be the exact complement; substantially equivalent sequences are acceptable.

From sequence data, it was observed that in allele 2 the guanine is substituted with an adenine at position 678 of the PCR product or at position 298 amino acid of the MC4R protein changing the aspartic acid codon (GAU) into an asparagine codon (AAU). The PCR test for the polymorphism used a forward primer of 5'-TGG CAA TAG CCA AGA ACA AG-3' (SEQ. ID NO: 6) and a reverse primer of 5'-CAG GGG ATA GCA ACA GAT GA-3' (SEQ. ID NO: 7). Pig specific primers used were a forward primer of 5'-TTA

AGT GGA GGA AGA AGG-3' (SEQ. ID NO: 8) and a reverse primer of 5'-CAT TAT GAC AGT TAA GCG G-3' (SEQ ID NO:9). The resulting amplified product of about 750 bp, when digested with *Taq I*, results in allelic fragments of 466, 225, and 76 bp (allele 1) or 542 and 225 bp (allele 2).

The marker may be identified by any method known to one of ordinary skill in the art which identifies the presence or absence of the marker, including for example, single-strand conformation polymorphism analysis (SSCP), RFLP analysis, heteroduplex analysis, denaturing gradient gel electrophoresis, and temperature gradient electrophoresis, ligase chain reaction or even direct sequencing of the MC4R gene and examination for the *Taq I* RFLP recognition pattern.

One or more additional restriction enzymes and/or probes and/or primers can be used. Additional enzymes, constructed probes, and primers can be determined by routine experimentation by those of ordinary skill in the art.

Other possible techniques include non-gel systems such as TaqMan™ (Perkin Elmer). In this system, oligonucleotide PCR primers are designed that flank the mutation in question and allow PCR amplification of the region. A third oligonucleotide probe is then designed to hybridize to the region containing the base subject to change between different alleles of the gene. This probe is labeled with fluorescent dyes at both the 5' and 3' ends. These dyes are chosen such that while in this proximity to each other the fluorescence of one of them is quenched by the other and cannot be detected. Extension by *Taq* DNA polymerase from the PCR primer positioned 5' on the template relative to the probe leads to the cleavage of the dye attached to the 5' end of the annealed probe through the 5' nuclease activity of the *Taq* DNA polymerase. This removes the quenching effect allowing detection of the fluorescence from the dye at the 3' end of the probe. The discrimination between different DNA sequences arises through the fact that if the hybridization of the probe to the template molecule is not complete, i.e., there is a mismatch of some form, the cleavage of the dye does not take place. Thus, only if the nucleotide sequence of the oligonucleotide probe is completely complementary to the template molecule to which it is bound will quenching be removed. A reaction mix can contain two different probe sequences each designed against different alleles that might be present, thus, allowing the detection of both alleles in on reaction.

Though the use of RFLPs is one method of detecting the polymorphism, other methods known to one of ordinary skill in the art may be used. Such methods include ones that analyze the polymorphic gene product and detect polymorphisms by detecting the resulting differences in the gene product.

Though the preferred method of separating restriction fragments is gel electrophoresis, other alternative methods known to one skilled in the art may be used to separate and determine the size of the restriction fragments.

It is possible to indirectly select for the polymorphism with alternative DNA markers. It is possible to establish a linkage between specific alleles of alternative DNA markers and alleles of DNA markers known to be associated with the MC4R gene which have previously been shown to be associated with a particular trait. Examples of markers on the published PiGMaP chromosome map which are linked to the MC4R gene include S0331, BHT0433, and S0313.

The reagents suitable for applying the methods of the present invention may be packaged into convenient kits. The kits provide the necessary materials, packaged into suitable containers. At a minimum, the kit contains a reagent that identifies the polymorphism in the MC4R gene that is associated with the traits of interest, fat content, growth rate, and feed consumption. Preferably, the reagent that identifies the polymorphism is a PCR set (a set of primers, DNA polymerase, and four nucleoside triphosphates) that hybridize with the MC4R gene or a fragment thereof. Preferably, the PCR set and restriction enzyme that cleaves the MC4R gene in at least one place are included in the kit. Preferably, the kit further comprises additional means, such as reagents, for detecting or measuring the detectable entity or providing a control. Other reagents used for hybridization, prehybridization, DNA extraction, visualization, and similar purposes may also be included, if desired.

The genetic markers, methods, and kits of the invention are useful in a breeding program to improve fat content, growth rate, and feed consumption characteristics in a breed, line, or population of animals. Continuous selection and breeding of animals that are at least heterozygous and preferably homozygous for the desired polymorphism associated with the particular trait would lead to a breed, line, or population having those desired traits. Thus, the marker is a selection tool.

The following examples are offered to illustrate, but not limit the invention.

EXAMPLE 1

Melanocortin 4 Receptor PCR-RFLP Test - *TaqI* polymorphism and Genetic Linkage Mapping of MC4R Gene

Primers:

Primers were designed from homologous regions of human and rat MC4R sequences (Genbank Accession No. s77415 and u67863, respectively). These primers were used to amplify a 750-bp sequence of the porcine MC4R gene.

MC4R1: 5' TGG CAA TAG CCA AGA ACA AG 3' (SEQ ID NO:6)
MC4R4: 5' CAG GGG ATA GCA ACA GAT GA 3' (SEQ ID NO:7)

PCR Conditions:

Mix 1:	10X Promega Buffer	1.0 μL
	25 mM $MgCl_2$	0.6 μL
	dNTPs mix (2.5mM each)	0.5 μL
	25 pmol/μL MC4R1	0.1 μL
	25 pmol/μL MC4R4	0.1 μL
	dd sterile H_2O	7.5 μL
	Taq Polymerase (5 U/μL)	0.07 μL
	Genomic DNA (12.5 ng/μL)	1.0 μL

Ten μL of Mix 1 and DNA were combined in reaction tube, then overlaid with mineral oil. The following PCR program was run: 94°C for 2 min.; 35 cycles of 94°C for 30 sec.; 58°C 1 min., and 72°C 1 min. 30 sec.; followed by a final extension at 72°C for 15 min.

Five μl of the PCR reaction product was checked on a standard 1% agarose gel to confirm amplification success and clean negative control. Product size is approximately 750 base pairs. Digestion was performed by the following procedure.

*Taq*I Digestion Reaction	10 μL reaction
PCR product	5.0 μL
10X *Taq*I NE Buffer	1.0 μL
BSA (10mg/ml)	0.1 μL
*Taq*I enzyme (20 U/μL)	0.5 μL
dd sterile H_2O	3.4 μL

A cocktail of the buffer, enzyme, BSA, and water was made. Five μL was added to each reaction tube containing the DNA. The mixture was then incubated at 65°C for at least 4 hours to overnight. Loading dye was mixed with the digestion reaction and the total volume was loaded on a 3% agarose gel. The major bands for allele 1 are about 466, 225, and 76 bp. The allele 2 genotype bands are 542 and 225 bp. The heterozygote genotype has both allele 1 and allele 2.

Results

The amplified PCR product is about 750 bp. The sequence of the PCR product confirmed that the PCR product is MC4R gene with 97.6%, and 92.2% identities at the amino acid and DNA level, respectively, to corresponding human sequences. (see Figs. 2 and 3).

The *Taq*I digestion of the PCR product produced allelic fragments of 466, 225, and 76 bp (allele 1), or 542 and 225 bp (allele 2). The heterozygote genotype has both types of alleles. Mendelian inheritance was observed in three three-generation international reference families, which were used to map this gene by linkage analysis.

The polymorphism between allele 1 and allele 2 resulting from a G → A transition at position 678 of the PCR product revealed a missense mutation of Aspartic acid codon (GAU) into Asparagine codon (AAU) at position 298 amino acid of MC4R protein. (See Figure 1, SEQ ID NO:1).

Allele frequencies were determined by genotyping of DNA samples from a small number of animals from different breeds (Table 1). Allele 1 was observed with a frequency of 1 in Meishan, but was not observed or observed at very low frequency in Hampshire and Yorkshire. The frequencies of allele 1 in Landrace and Chester White were 0.5, respectively.

Figures 2 and 3 illustrate the differences between the DNA and amino acid sequences of the human and porcine MC4R gene (SEQ ID NOS:2-5).

TABLE 1

The Frequency of Allele 1 in Different Pig Breeds

Breed	# Animals	Freq. Allele 1
Meishan	8	1
Large White	8	0.56
Yorkshire	6	0.08
Hampshire	5	0
Landrace	4	0.5
Chester White	4	0.5
Minzu	2	1
Wild Boar	2	1

Linkage Analyses

Two-point and multi-point linkage analyses were performed on the genotypes of international reference families. See Figs. 4a-4c. The data were analyzed by using the CRI-MAP program. MC4R was significantly linked to several markers on porcine chromosome (SSC) 1. The most closely linked markers (recombination fraction and LOD score in parentheses) are SO331 (0.02, 21.97), BHT0433 (0.02, 21.32), and SO313 (0.00, 17.76) by two-point linkage analysis. A multi-point linkage analysis produced the best map order of markers and MC4R (with distance in Kosambi cM): KGF-5.8-CAPN3-2.5-MEF2A-6.1-MC4R-5.6-SO313.

Somatic cell hybrid panel of pig and rodent was used to assign MC4R to a cytogenetic region. PCR products from pig specific primers were amplified in clones 7, 8, 16, 18, and 19. MC4R was localized to SSC1q 22-27.

EXAMPLE 2

MC4R Receptor PCR-RFLP Test using Pig Specific Primers and Genetic Linkage Mapping of the Porcine MC4R Gene

Pig Specific Primer Sequences

Forward primer: 5'-TTA AGT GGA GGA AGA AGG-3' (SEQ ID NO:8)

Reverse primer: 5'-CAT TAT GAC AGT TAA GCG G-3' (SEQ ID NO:9)

Method of Detection

The PCR reaction was performed using

Porcine genomic DNA	12.5 ng
1x PCR buffer	
$MgCl_2$	1.5 mM
dNTP	0.125 mM
Forward primer	0.3 μM
Reverse primer	0.3 μM
Taq DNA polymerase (Promega)	0.35 U

in a 10 μL final volume. The PCR profile included 2 min. at 94°C; 35 cycles of 30 sec. at 94°C, 1 min. at 56°C, 1 min. 30 sec. at 72°C; and 15 min. at 72°C in a Robocycler (Statagene, La Jolla, CA). A 5.0 μL aliquot of the PCR products was digested in a total volume of 10 μL with 10 U of *Taq*I incubated overnight at 65°C. The digestion products were electrophoresed on a 3% agarose gel.

Description of Polymorphism

The *Taq*I digestion of the PCR product produced fragments of 466, 225, and 76 bp in allele 1 and 542 and 225 bp in allele 2. The heterozygous genotype has fragments of both allele 1 and allele 2.

Pattern of Inheritance

Autosomal segregation of Mendelian inheritance was observed in three three-generation European PiGMaP families (Archibald et al., 1995).

Allele Frequencies

Allele frequencies were determined by genotyping the grandparental animals of the European PiGMaP families and unrelated animals from ISU reference families. Allele 1 was observed with the following frequencies.

TABLE 2

The Frequency of Allele 1 in Different Pig Breeds

Breed	# Animals	Freq. Allele 1
Meishan	8	1
Large White	8	0.56
Yorkshire	10	0.15
Hampshire	12	0
Landrace	8	0.56
Chester White	8	0.56
Minzu	2	1
Wild Boar	2	1

Chromosomal Location

Two-point and multi-point linkage analysis were performed on the genotypes of three PiGMaP families using the CRI-MAP program (Green et al. 1990). MC4R was

significantly linked to several markers on porcine chromosome 1 (SSC 1). The most closely linked markers (recombination fraction and LOD score in parentheses) are SO331 (0.02, 21.97), BHT0433 (0.02, 21.32), and SO313 (0.00, 17.76) according to two-point linkage analysis. The best map order of MC4R with respect to other linked markers produced by multi-point linkage analysis is as follows (with distance in Kosambi cM): KGF-5.8-CAPN3-2.5-MEF2A-6.1-MC4R-5.6-SO313.

Comments

The Melanocortin-4 Receptor is a G protein-coupled, seven-transmembrane receptor expressed in the brain. Huszar et al. (1997) found that inactivation of MC4R gene resulted in a maturity onset obesity syndrome in mice and demonstrated a major role of MC4R protein in the regulation of energy balance. The MC4R gene has been mapped to human chromosome 18q21.3 (Gantz et al., 1993). The localization of MC4R gene to SSC 1 is consistent with previous chromosome painting data indicating synteny between this chromosome and HSA 18 and 15 (Goureau et al., 1996). However, the gene order of several genes previously mapped from HSA 18 and 15 to SSC 1, including CAPN3, KGF, and MEF2A, is not conserved with MC4R. Therefore, mapping of MC4R to SSC 1 may identify an evolutionary breakpoint between HSA 18 and 15 in relation to SSC 1.

EXAMPLE 3

Association of Marker with Enhanced Metabolic Characteristics

In a preliminary study to determine which allele is associated with which trait and in which breeds, the genotypes of several lines of animals were correlated with days to 110 kg, backfat measurements, daily gains, and average daily feed intake. The pigs used in the study were from lines from Pig Improvement Company (PIC).

Data was accumulated using the PCR test described *supra* for the 1 and 2 allele of the MC4R gene. The collected data is summarized in Tables 3-8 below.

According to the results, allele 1 is the significantly leaner allele (see P2 backfat measurements) in all lines except in Chinese pigs where it is the fat allele. Allele 2 is

associated with significantly faster rate of gain (test daily gain) in the tested commercial lines. Overall allele 1 is associated with lower feed intake.

TABLE 3

Number of observations

MC4R genotype	L02	L03	L19	L65	Overall	L95
11	88	30		32	150	20
12	57	54	56	74	241	67
22	12	31	254	33	330	37
				Total	721	

MC4R genotype:

11 = homozygous allele 1

12 = heterozygous

22 = homozygous allele 2

TABLE 4

Number of observations (males/females)

MC4R genotype	L02	L03	L19	L65	Overall		L95
11	9/79	12/18		15/17	36/114		0/20
12	9/48	37/17	12/44	44/30	102/139		0/67
22	3/9	28/3	89/165	21/12	141/189		0/37

TABLE 5

Days to 110kg

MC4R genotype	L02	L03	L19	L65	Overall		L95
11	169.7	172.4		169.6	168.5		219.1
12	170.2	171.5	165.0	171.2	168.7		212.2
22	165.3	173.4	162.9	170.3	167.1		211.4
	P<.23	P<.75	P<.15	P<.76	P<.31		P<.27

TABLE 6

P2 backfat (mm)

MC4R Genotype	L02	L03	L19	L65	Overall		L95
11	10.8	11.9		9.7	11.1		22.8
12	11.3	12.5	12.2	10.5	11.8		21.5
22	12.1	12.7	12.6	10.7	12.1		20.3
	P<.10	P<.43	P<.34	P<.17	P<.006		P<.17

TABLE 7

Test daily gain (gm/d)

MC4R genotype	L02	L03	L19	L65	Overall		L95
11	882.2	811.0		881.8	871.9		688.8
12	891.2	820.5	875.6	873.0	876.3		676.2
22	969.1	819.5	906.7	906.2	906.9		692.5
	P<.01	P<.96	P<.05	P<.24	P<.006		P<.66

TABLE 8

Average daily feed intake (kg/d), boars only, except L95 which was gilts only

MC4R genotype	L02	L03	L19	L65	Overall		L95
11	2.31	1.78		1.75	1.89		2.05
12	2.11	1.90	1.97	1.90	1.96		2.03
22	2.15	1.97	2.00	1.97	2.02		2.08
	P<.84	P<.14	P<.56	P<.14	P<.16		P<.36

EXAMPLE 4

A Missense Variant of the Porcine Melanocortin-4 Receptor (*MC4R*) Gene is Associated with Fatness, Growth, and Feed Intake Traits

To determine if there was an association of this *MC4R* polymorphism with phenotypic variation the mutation was tested in a large number of individual animals from several different pig lines. Analyses of growth and performance test records showed significant associations of *MC4R* genotypes with backfat, growth rate and feed intake in a number of lines. It is probable that the variant amino acid residue of the *MC4R* mutation causes a significant change of the MC4R function. These results support the functional significance of a pig *MC4R* missense mutation and suggest that comparative genomics based on model species may be equally important for application to farm animals as they are for human medicine.

Identification of mutations in the *leptin* and the *leptin receptor* has provided some information on genetic components involved in the regulation of energy balance (Zhang et al. 1994; Tartaglia et al. 1995). Genetic studies using animal models have facilitated the identification of major genetic causes of obesity (Andersson 1996; Pomp 1997; Giridharan 1998). Furthermore, several other genes involved in the neural signaling pathway of energy homeostasis have been identified (Flier and Maratos-Flier 1998; Schwartz et al. 1999). Of particular interest among candidate signaling molecules involved in the

regulation of energy homeostasis is the melanocortin-4 receptor (MC4R). The MC4R response to leptin signaling is a link between food intake and body weight (Seeley et al. 1997; Marsh et al. 1999). Neuropeptide Y (NPY) signaling in the central nervous system is also mediated by the MC4R protein (Kask et al. 1998). Several mutations in MC4R including frameshift and nonsense mutations are associated with dominantly inherited obesity in humans (Vaisse et al. 1998; Yeo et al. 1998). Some other MC4R missense mutations in humans have also been identified (Gotoda et al. 1997; Hinney et al. 1999) but the functional significance of these mutations has not been characterized.

Selection based on growth characteristics has been of great importance to the pig industry because of costs associated with feeding and consumer preference for lean meat. Efficient genetic improvement in these quantitative traits may be augmented through the use of marker assisted selection (MAS) using high density genetic maps (Dekkers and van Arendonk 1998; Rothschild and Plastow 1999). An important tool in this process is comparative mapping using the well-developed human and mouse gene maps, which assist in the identification of corresponding genomic regions or major genes controlling growth and performance traits in the pig. Biological understanding of complex traits in human or model species offers an alternative approach to identify genes responsible for the traits of economic interest in livestock. Several quantitative trait loci (QTL) linkage scans using phenotypically divergent breeds and candidate gene analyses have been successfully conducted for fatness and growth traits (Yu et al. 1995; Casas-Carrillo et al. 1997; Knorr et al. 1997; Knott et al. 1998; Rohrer et al. 1998; Wang et al. 1998; Paszek et al. 1999), but no individual genes with major effects on growth and performance traits have yet been established for commercial populations. The role of MC4R in feed intake and obesity suggests it may be an important genetic marker for the growth-related traits in the pig.

Materials and Methods

Animals. Pigs were raised under normal production conditions under the care of PIC employees in nucleus farms in the United States and Europe. Pigs were put on the performance test at approximately 70 days of age and taken off test after 13 weeks. At the end of the trial backfat was measured ultrasonically in real time (B mode) at the 10th rib 2 cm from the centerline. Average daily gain (growth) over the test period was calculated as

weight gained divided by days on test. Days to 110 kg market weight was estimated using standard procedures and feed intake was measured using individual electronic measurement equipment.

PCR amplification of a pig MC4R gene fragment. Primers were designed from homologous regions of human and rat MC4R sequences (GenBank accession no. s77415 and u67863, respectively). The primers were: forward primer: 5'-TGG CAA TAG CCA AGA ACA AG-3' (SEQ. ID NO:6) and reverse primer: 5'-CAG GGG ATA GCA ACA GAT GA-3' (SEQ. ID NO:7). The PCR reaction was performed using 12.5 ng of porcine genomic DNA, 1x PCR buffer, 1.5 mM $MgCl_2$, 0.125 mM dNTPs, 0.3 mM of each primer, and 0.35 U *Taq* DNA polymerase (Promega) in a 10μL final volume. The conditions for PCR were as follows: 2 min at 94°C; 35 cycles of 30 s at 94°C, 1 min at 56°C, 1 min 30 s at 92°C, and a final 15 min extension at 72°C in a Robocycler (Stratagene, La Jolla, CA).

Sequencing and mutation detection. Sequencing of the PCR products from several individual pigs of different breeds was conducted and the sequences were compared to detect any nucleotide change. Sequencing was performed on an ABI sequencer 377 (Applied Biosystems). The porcine MC4R sequence has been submitted to GenBank, and has accession number AF087937. The sequence analysis revealed one nucleotide substitution situated within a *TaqI* restriction enzyme recognition site (Kim et al. 1999). A set of primers was then designed to generate a smaller MC4R gene fragment, which contained only one informative *TaqI* restriction site to specify the polymorphic site and to facilitate the PCR-RFLP test. These primers were: forward 5'-TAC CCT GAC CAT CTT GAT TG-3' (SEQ. ID NO:10) and reverse: 5'-ATA GCA ACA GAT GAT CTC TTT G-3' (SEQ. ID NO:11).

Statistical analysis. Analysis of variance procedures were used with a mixed model that accounted for the fixed effects of farm, test period, sex of the animal, the MC4R genotype and site (random). All animals in lines of American/European descent (Lines A-D) were pooled for the overall analysis and in this analysis line of origin was included.

Mean effects were estimated for each genotype and are presented in Tables 9-15. Overall F tests were used to determine level of significance.

Results

Identification of a missense mutation in the pig MC4R gene. The MC4R gene consists of approximately 1 kb of coding sequence contained within a single exon. About 750 bp of a pig MC4R gene fragment was produced by PCR (Kim et al. 1999). The sequence of the PCR product confirmed that the PCR product is the MC4R gene with 92.2% and 97.6% identities at nucleotide and the amino acid levels, respectively, to the human MC4R sequence. Multiple alignments of the sequences from individual animals of several breeds identified a single nucleotide substitution (G→A; Fig. 5). The polymorphism revealed a missense mutation that replaces aspartic acid (GAU) with asparagine (AAU) at the position identical to amino acid 298 of human MC4R protein. To confirm this base change, we designed pig-specific primers flanking the polymorphic site and analyzed the polymorphism as a *TaqI* PCR-RFLP gel (Fig. 6). Figure 6 shows a *TaqI* digestion of the PCR product analyzed by agarose-gel electrophoresis. Allele 1 produced 156 and 70 bp fragments and allele 2 produced a 226 bp fragment as the PCR-RFLP. The heterozygote has both allele 1 and 2 fragments. Molecular marker (M) and MC4R genotypes are indicated at the top of each lane.

The MC4R missense mutation is within a highly conserved region among melanocortin receptors (MCR). The MCR is a subfamily of G-protein coupled receptors (GPCR) containing certain conserved structural elements common to most other GPCRs, but overall amino acid identities between MCR and other GPCRs are low (Tatro 1996). A multiple-alignment of the predicted amino acid sequences of the pig MC4R with MC4R proteins from other species, other MCR proteins, or representative GPCRs showed that the aspartic acid found at position 298 of the seventh transmembrane domain is very highly conserved in the MCR proteins (Fig. 7). It is interesting to note, however, that this position is occupied by asparagine in most other GPCRs. The MCR proteins show 40-80% amino acid identity with each other (Tatro 1996), but the second intracytoplasmic loop and the seventh transmembrane domain are highly conserved among MCR proteins (Gantz et al.

1993). Some of the relationships between MCR structure and function have been discovered by the studies of natural and experimental mutations in humans and mice (Robbins et al. 1993; Valverde et al. 1995; Frandberg et al. 1998). These studies indicate that some mutations in highly conserved regions cause structural changes and alter the function of the receptor. The Asp298Asn substitution mutation could have an effect on the function of the receptor. However, this will require further testing but it is known that change of the homologous residue in MC1R (Asp294His) is associated with fair skin and red hair in humans (Valverde et al. 1995).

<u>The MC4R missense mutation is associated with obesity-related traits.</u> To investigate the effects of the missense mutation, the relationship of MC4R genotypes was analyzed for the effects on variation in growth rate, backfat, and feed consumed in over 1,800 animals from several commercial pig lines from PIC, an international pig breeding company. The animals were from closed commercial lines of European/American breeds (Lines A-D) together with a line originating from a cross between a European and a Chinese breed (Line E). In lines A-D significant associations of the MC4R genotypes were found for all performance traits. The animals homozygous for allele 1 had on average significantly less backfat ($P < .001$), lower daily gain ($P < .001$), and lower feed intake($P < .01$) than those of the homozygous 22 genotype animals (Tables 11, 13, & 15). Overall, pigs with the 11 genotype had approximately 9% less backfat than pigs with the 22 genotype (Table 11), whereas pigs with the 22 genotype grow significantly faster (37g/day) than pigs with the 11 genotype (Table 13). These results appear to be a function of appetite because the 22 genotype animals consume considerably more feed (Table 15). The association between the missense variant of the MC4R gene and related performance traits is clearly established in European/American breeds. Although the number of tested animals is much smaller, these results were not seen in the considerably fatter Chinese crossed line (Line E). Interestingly, line E shows a trend for backfat in the opposite direction to that observed in the other lines (Table 11).

Discussion

The present study clearly demonstrates that the porcine MC4R missense mutation is significantly associated with several performance traits in pigs. Allele 1 representing Asp298, the well conserved amino acid within other MCR subtypes and other species MC4R, was associated with less backfat thickness, slower growth rate, and lower feed intake and allele 2 representing Asn298 was associated with fatter, higher feed intake, and faster growing animals. As the highly conserved residues in the melanocortin receptor proteins have important roles for ligand binding or intracellular signal transmission (Tatro 1996), the MC4R variants might exert functionally distinct abilities in the regulation of food intake and body weight. Further testing of this hypothesis will provide important insights into the structural basis of MCR function and a molecular target for the treatment of human obesity.

Allele 1 was associated with the fattest animals in Line E, which was derived by crossing a Chinese Large White breed with a line of Meishan origin. This is surprising given that the mutation causes a significant amino acid change in a well-conserved region. The result may be due to sampling. However, if we assume that this result will be significant when more results are added there are several possible explanations. One possibility could be the difference in the background gene effects (epistasis). As growth and fatness are complex polygenic traits, it is certainly possible that the Chinese breed has some distinct allelic interactions derived from several hundred years of isolation and these putative interaction(s) might create variation in polygenic traits within crosses between widely different lines (Frankel and Schork 1996). Several QTL analyses have been conducted for fatness and growth traits using divergent lines (Cases-Carrillo et al. 1997; Knott et al. 1998; Rohrer et al. 1998; Wang et al. 1998; Paszek et al. 1999), but QTL have not been reported near the C4R locus, which maps to chromosome 1 at approximately 80 cm on the linkage map (data not shown). It may mean that the epistatic effects of the MC4R alleles suggested in Line E have made it difficult to observe the MC4R locus in most QTL experiments which have involved crosses between Chinese and European/American lines. It is likely that the effect of some alleles will be variable in the different backgrounds and hard to detect in QTL experiments involving genetically divergent breeds.

The effect of MC4R variant will possibly be explained by further studies on the biological effect caused by this mutation in other pig breeds and lines. However, given the strong relationship of MC4R variants to leanness, growth and feed intake, this mutation could be used immediately for merker assisted selection (Meuwissen and Goddard 1996) to develop lines of pigs to satisfy particular customer requirements. For instance, in sow lines where appetite is normally decreased after farrowing, selection for the MC4R 2 allele could help improve feed intake. Furthermore, in some lines deemed to be too fat, selection for allele 1 could be employed and in lines that were considered too slow growth allele 2 selection could be also employed. Therefore, genotyping for the MC4R mutation in pig breeding lines will improve the selection efficiency of feed related production traits including growth and leanness. The candidate gene approach has also been used for investigating the role of the porcine leptin gene (Jiang and Gibson 1999). However, in the leptin case, although there was evidence for an association between a leptin polymorphism and backfat depth in a cross between a commercial breed and an unimproved line, there was no clear association in the different commercial lines tested (Jiang and Gibson 1999). Therefore, it should not be assumed that since one finds a gene that one can assume a relationship exists. In contrast, with MC4R we have determined that variation in this candidate gene can explain significant variation for backfat, growth rate, and feed intake in commercial lines of pigs. These results with MC4R illustrate the potential value of comparative genetic analyses using candidate genes in livestock genomics.

EFFECT OF MC4R GENOTYPE ON SEVERAL PRODUCTION TRAITS IN THE PIG

TABLE 9

Number of observations (males/females/totals) for Days to 110 kg and backfat

MC4R	LINE A	LINE B	LINE C	LINE D	Total	Line E
11	9/212/221	12/94/106		37/17/54	58/323/381	0/20/20
12	9/150/159	37/96/133	12/158/170	152/30/182	210/434/644	0/67/67
22	3/16/19	28/36/64	89/356/445	155/12/167	275/420/695	0/37/37

TABLE 10

Days to 110kg

MC4 R	LINE A	LINE B	LINE C	LINE D	Total	Line E
11	166.3+/-0.8	168.4+/-1.4		170.0+/-2.4	167.9+/-0.9	219.1+/-4.8
12	165.6+/-0.9	166.8+/-1.1	163.9+/-1.0	170.2+/-1.8	166.9+/-0.8	212.2+/-3.4
22	162.3+/-2.3	166.8+/-1.5	161.5+/-0.8	167.0+/-1.9	164.6+/-0.9	211.4+/-4.0
	P <.24	P <.47	P <.007	P <.10	P <.001	P <.27

TABLE 11

10th rib Backfat (mm)

MC4 R	LINE A	LINE B	LINE C	LINE D	Total	Line E
11	10.7+/-0.2	12.1+/-0.2		9.8+/-0.5	11.1+/-0.2	22.8+/-1.2
12	11.2+/-0.2	12.5+/-0.2	12.3+/-0.2	10.5+/-0.4	11.6+/-0.2	21.5+/-0.9
22	12.5+/-0.5	12.6+/-0.3	12.7+/-0.2	10.9+/-0.4	12.0+/-0.2	20.3+/-1.0
	P <.02	P <.31	P <.06	P <.05	P <.001	P <.17

TABLE 12

Number of observations (males/females/totals for Test daily gain

MC4 R	LINE A	LINE B	LINE C	LINE D	Total	Line E
11	9/105/114	12/38/50		37/17/54	58/160/218	0/20/20
12	9/65/74	37/35/72	12/97/109	152/30/182	210/227/437	0/67/67
22	3/13/15	28/15/43	89/225/314	155/12/167	275/265/539	0/37/37

TABLE 13

Test daily gain (gm/day)

MC4R	LINE A	LINE B	LINE C	LINE D	Total	Line E
11	892.6+/-10.4	841.7+/-13.8		882.2+/-18.4	871.9+/-10.2	688.8+/-24.5
12	913.3+/-11.6	868.4+/-12.1	882.2+/-12.9	883.7+/-14.3	885.1+/-8.9	676.2+/-17.6
22	982.8+/-22.8	862.4+/-15.1	913.4+/-10.5	904.6+/-15.1	908.8+/-9.3	692.5+/-20.4
	P <.001	P <.28	P <.006	P <.20	P <.001	P <.66

TABLE 14

Number of observations (males/females/total) for average daily feed intake

MC4R	LINE A	LINE B	LINE C	LINE D	Total	Line E
11	7/0/7	11/0/11		13/0/13	31/0/31	0/18/18
12	8/0/8	31/0/31	9/0/9	34/0/34	82/0/82	0/63/63
22	3/0/3	25/0/25	74/0/74	16/0/16	118/0/118	0/32/32

TABLE 15

Average daily feed intake (kg/day), boars only except LINE E which was gilts only

MC4R	LINE A	LINE B	LINE C	LINE D	Total	Line E
11	2.31+/-0.2	1.78+/-0.09		1.75+/-0.06	1.94+/-0.07	2.05+/-0.10
12	2.11+/-0.3	1.90+/-0.07	1.97+/-0.10	1.90+/-0.07	2.03+/-0.06	2.03+/-0.07
22	2.15+/-0.4	1.97+/-0.06	2.00+/-0.07	1.97+/-0.08	2.11+/-0.06	2.08+/-0.08
	P <.84	P <.14	P <.56	P <.14	P <.01	P <.36

Having described the invention with reference to particular compositions, theories of effectiveness, and the like, it will be apparent to those of skill in the art that it is not intended that the invention be limited by such illustrative embodiments or mechanisms, and that modifications can be made without departing from the scope or spirit of the

invention, as defined by the appended claims. It is intended that all such obvious modifications and variations be included within the scope of the present invention as defined in the appended claims. The claims are meant to cover the claimed components and steps in any sequence which is effective to meet the objectives there intended, unless the context specifically indicates to the contrary.

The following citations are hereby incorporated in their entirety by reference:

Andersson LB (1996) "Genes and obesity", Ann Med., 28:5-7.

Arden KC (1990). "The Receptors for Prolactin and Growth Hormone are Localized in the same Region of Human Chromosome 5", Cytogenet. Cell Genet. 53:161-165.

Barinaga M (1996). "Researchers Nail Down Leptin Receptor", Science 271:913.

Bray GA (1978). Physiol. Rev. 59, 719-809.

Casas-Carillo E (1997) "Mapping genomic regions associated with growth rate in pigs", J Anim Sci 75:2047-2053.

Chajlani V, "Molecular Cloning of a Novel Hum. n Melanocortin Receptor", Biochem. Biophys. Res. Commun., 195, 866-873.

Chen H (1995). "Evidence that the diabetes gene encodes the leptin receptor: Identification of a mutation in the leptin receptor gene in db/db mice", Cell 84, 491-495.

Chevalet C (1996). CABIOS. In Press.

Chua SC (1996). Science 271, 994-996.

Chung WK (1996). Genome Res. 6, 431-438.

Cioffi JA (1996). Nature Med. 2, 585-589.

Cybulsky MI (1991). "Gene Structure, Chromosomal Location, and Basis for Alternative mRNA Splicing of the Human VCAM1 Gene". Proc. Nat. Acad. Sci. 88:7859-7863.

Dekkers JCM (1998) "Optimizing selection for quantitative traits with information on an identified locus in outbred population", Genet Res 71:257-275.

Flier JS (1998), "Obesity and the hypothalamus: Novel peptides for new pathways", Cell 92:437-440.

Frandberg P (1997) "Glutamine^{235} and argine^{272} in human melanocortin 5 receptor determines its low affinity to MSH", Biochem Biophys Res Commun 236:489-492.

Frankel WN (1996) "Who's afraid of epistasis?" Nat Genet 14:371-373.

Gantz I (1993a) "Molecular Cloning of a Novel Melanocortin Receptor", J. Biol. Chem. 268, 8246-8250.

Gantz (1993) "Molecular Cloning, Expression in Gene Localization of a Fourth Melanocortin Receptor", J. Biol. Chem. 268, 15174-15179.

Gantz (1994) "Molecular Cloning Expression and Characterization of a Fifth Melanocortin Receptor", Biochem. Biophys. Res. Comm., 200, 1214-1220.

Giridharan NV (1998) "Animal models of obesity and their usefulness in molecular approach to obesity", Indian J Med res 108:225-242.

Gotoda T (1997) "Molecular screening of the human melanocortin-4 receptor gene: identification of a missense variant showing no association with obesity, plasma glucose, or insulin" Diabetologia 40:976-979.

Goureau A (1996). Genomics. In Press.

Griffon N (1994) "Molecular Cloning and Characterization of the Rat Fifth Melanocortin Receptor", Biochem. Biophys. Res. Comm., 200, 1007-1014.

Helm J (1994) "Rapid Communication: SacI Restriction Fragment Length Polymorphism with a Porcine Vascular Cell Adhesion Molecule 1 (VCAM1) cDNA Fragment", J. Anim. Science 72:2764.

Hinney A (1999) "Several mutations in the melanocortin-4 receptor gene including a nonsense and a frameshift mutation associated with dominantly inherited obesity in humans" J Clin Endocrinol Metab 84:1483-1486.

Huszar (1997) "Targeted disruption of the Melanocortin-4 Receptor Results in /Obesity in Mice", Cell 88:131-141.

Kask A (1998) "Evidence that orexigenic effects of melanocortin 4 receptor antagonist HS014 are mediated by neuropeptide Y" Biochem Biophys Res Commun 248:245-249.

Kim KS (1999) "Rapid Communication: Linkage and physical mapping of the porcine Melanocortin-4 Receptor (MC4R gene" J Anim Sci (submitted).

Knorr C (1997) "Associations of GH gene variants with performance traits in F2 generations of European wild boar, Pietrain and Meishan pigs" Anim Genet 28:124-128.

Knott SA (1998) "Multiple marker mapping of quantitative trait loci in a cross between outbred wild boar and large white pigs" Genetics 149:1069-1680.

Labbe O (1994) "Molecular Cloning of a Mouse Melanocortin-5 Receptor Gene Widely Expressed in Peripheral Tissues", Biochemistry, 33, 4543-4549.

Lee GH (1996). Nature 379, 632-635.

Lu Dongsi "Agouti Protein is an Antagonist of the Melanocyte-Stimulating Hormone Receptor" Letters to Nature.

Marsh DJ (1999) "Response of melanocortin-4 receptor-deficient mice to anorectic and orexigenic peptides" Nat Genet 21:119-122.

Messer L, (1996a) "Mapping and Investigation of Candidate Genes for Litter Size in French Large White Pigs" ISAG Proceedings (submitted).

Messer L (1996b) "Linkage Mapping of the Retinoic Acid Receptor Gamma (RARG) Gene to Porcine Chromosome 5". Anim. Genet. (in press).

Messer L (1996c) "Linkage mapping of the Retinol Binding Protein 4 (RBP4) Gene to Porcine Chromosome 14". Mammal. Genome (in press).

Meuwissen THE (1996) "The use of marker haplotypes in animal breeding schemes" Genet Sel Evol 28:161-176.

Montjoy KG (1992) "The Cloning of a Family of Genes that Encode the Melanocortin Receptors", Science 257, 1248-1251.

Montjoy KG (1994) "Localization of the Melanocortin-4 Receptor (MC4-R) In Neuroendocrine In Autonomic Control Circuits in the Brain" Mol. Endocrinol. 8, 1298-1308.

Paszek A (1996) "Genomic Scan for Quantitative Trait Loci in Swine." Proceed. Midwest. ASAS Meeting, p. 24.

Paszek AA (1999) "Interval mapping of growth in divergent swine cross" Mamm Genome 10:117-122.

Phillips M (1996) "Leptin Receptor Missense Mutation in the Fatty Zucker Rat", Nature Genetics 13:18-19.

Pomp D (1997) "Genetic dissection of obesity in polygenic animal models" Behav Genet 27:285-306.

Robbins LS (1993) "Pigmentation phenotypes of variant extension locus alleles result from point mutations that alter MSH receptor function" Cell 72:827-834.

Robic A (1996). Mamm. Genome 7, 438-445.

Rohrer GA (1998) "Identification of quantitative trait loci affecting carcass composition in swine: I. Fat deposition traits" J Anim Sci 76:2247-2254.

Rothschild MF (1996). "The Estrogen Receptor Locus is Associated With a Major Gene for Litter Size in Pigs". PNAS 93: 201-205.

Rothschild MF(1999) "Advances in pig genomics and industry applications" AgBiotechNet 1:1-8.

Schwartz MW (1999) "Model for the regulation of energy balance and adiposity by the central nervous system" Am J Clin Nutr 69:584-96.

Seeley RJ (1997) "Melanocortin receptors in leptin effects", Nature 390:349.

Tartaglia LA (1995) "Identification and Expression Cloning of a Leptin Receptor" Cell 83:1263-1271.

Tatro JB (1996) "Receptor biology of the melanocortins, a family of neuroimmunomodulatory peptides", Neuroimmunomodulation 3:259-284.

Truett GE (1995). Mamm. Genome 6, 25-30.

Vaisse C (1998) "A frameshift mutation in human MC4R is associated with a dominant form of obesity" Nat Genet 20:113-114.

Valverde P (1995) "Variants of the melanocyte-stimulating hormone receptor gene are associated with red hair and fair skin in humans" Nat Genet 11:328-330.

Wang L (1998) "A direct search for quantitative trait loci on chromosomes 4 and 7 in pigs" J Anim Sci 76:2560-2567.

Warner CM (1991). "The Swine Major Histocompatibility Complex (SLA)" in Immunogenetics of the MHC, VCH Publishers, NY, NY, pp. 368-397.

Winick JD (1996). Genomics 36, 221-222.

Xy Y (1994) "Identification of the Ped Gene at the Molecular Level: The Q9 MHC Class I Transgene Converts the Ped Slow Phenotype to Ped Fast Phenotype" Biol. Reprod. 51:695-699.

Yeo GS (1998) "A frameshift mutation in MC4R associated with dominantly inherited human obesity" Nat Genet 20:111-112.

Yerle M (1996). Cytogenet. Cell Genet. 73, 194-202.

Youngs CR (1993) "Investigation into the Control of Litter Size in Swine: I. Comparative Studies on *in vitro* Development of Meishan and Yorkshire Preimplantation Embryos" J. Anim. Sci., 1561-1565.

Yu TP (1995) "Association of PIT1 polymorphisms with growth and carcass traits in pigs" J Anim Sci 73:1282-1288.

Zhang Y (1994) "Positional cloning of the mouse obese gene and its human homologue", Nature 372:425-432.

Ziang ZH (1999) "Genetic polymorphisms in the leptin gene and their association with fatness in four pig breeds" Mamm Genome 12:191-193.

What is claimed is:

1. A method of identifying an animal which possesses a genotype indicative of the metabolic traits of fat content, growth rate, and feed consumption, the method comprising:

a) obtaining a nucleic acid sample from the animal, and

b) identifying a polymorphism in the MC4R gene of the sample.

2. The method of claim 1 wherein the polymorphism is characterized by a nucleotide position 678 of the PCR product of the MC4R gene.

3. The method of claim 1 wherein the animal is a pig.

4. The method of claim 2 wherein the polymorphism at the nucleotide position 678 is associated with fat content.

5. The method of claim 2 wherein a guanine at the nucleotide position 678 is associated with lower feed intake.

6. The method of claim 2 wherein an adenine at the nucleotide position 678 is associated with a faster rate of gain.

7. The method of claim 1 wherein the step of identifying the polymorphism is a method employing allele specific oligonucleotides.

8. The method of claim 1 wherein the step of identifying the polymorphism is selected from the group consisting of restriction fragment length polymorphism (RFLP) analysis, heteroduplex analysis, single strand conformational polymorphism (SSCP) analysis, denaturing gradient gel electrophoresis (DGGE),temperature gradient gel electrophoresis (TGGE), and use of linked genetic markers.

9. The method of claim 8 wherein the step of identifying the polymorphism comprises RFLP analysis.

10. The method of claim 1 further comprising the step of amplifying the MC4R gene sequence.

11. The method of claim 10 further comprising the step of digesting the amplified region with the restriction endonuclease *Taq I.*

12. The amplified gene sequence of claim 10 wherein primers used in the amplification are selected from the group consisting of SEQ. ID NO:6, SEQ. ID NO:7, SEQ. ID NO:8, SEQ. ID NO:9, SEQ. ID NO:10, and SEQ. ID NO:11.

13. A single strand of an oligonucleotide primer useful for detecting nucleotide 678 of the PCR product of a MC4R gene the primer consisting of a nucleotide sequence having about 4-30 contiguous bases from SEQ ID NO:1.

14. The oligonucleotide of claim 13 wherein the oligonucleotide has the nucleotide sequence represented by SEQ ID NO:6.

15. The oligonucleotide of claim 13 wherein the oligonucleotide has the nucleotide sequence represented by SEQ ID NO:7.

16. The oligonucleotide of claim 13 wherein the oligonucleotide has the nucleotide sequence represented by SEQ ID NO:8.

17. The oligonucleotide of claim 13 wherein the oligonucleotide has the nucleotide sequence represented by SEQ ID NO:9.

18. The oligonucleotide of claim 13 wherein the oligonucleotide has the nucleotide sequence represented by SEQ ID NO:10.

19. The oligonucleotide of claim 13 wherein the oligonucleotide has the nucleotide sequence represented by SEQ ID NO:11.

20. A method of identifying an animal which possess a desired genotype indicative of the metabolic traits of fat content, growth rate, and feed consumption, the method comprising

a) obtaining a sample of genomic DNA,

b) digesting the sample with *Taq I* to obtain fragments,

c) separating the fragments obtained from the digestion, and

d) identifying the presence or absence of a *Taq I* site at base 678 of the PCR product of the MC4R gene.

21. The method of claim 20 further comprising the step of selecting animals with the desired genotype for breeding.

22. The method of claim 20 wherein the site is identifiable by fragments of 466, 225, and 76 bp when a guanine is present at base 678 and fragments of 542 and 225 bp when an adenine is present when a restriction enzyme which cuts at the same recognition site as *Taq I* is used.

23. The method of claim 20 wherein the step of identifying comprises detecting the *Taq I* site by amplification.

24. A kit for evaluating a sample of animal DNA comprising a reagent in a container that identifies a polymorphism in a MC4R gene.

25. The kit of claim 24 wherein the reagent is a primer that amplifies the MC4R gene or a fragment thereof.

26. The kit of claim 24 further comprising a DNA polymerase which cleaves the MC4R gene, a forward primer, and a reverse primer, wherein the primers are capable of amplifying a region of the MC4R gene which contains a polymorphic site.

27. A primer for assaying the presence of a polymorphic *TaqI* site in the MC4R gene wherein the primer comprises a sequence selected from the group consisting of SEQ. ID NO:6, SEQ. ID NO:7, SEQ. ID NO:8, SEQ. ID NO:9, SEQ. ID NO:10, and SEQ. ID NO:11.

28. A method for selecting animals for the desired traits of lower fat content, faster growth rate, or lower feed consumption comprising the steps of

a) obtaining a nucleic acid sample from an animal,

b) identifying a polymorphism characterized by a nucleotide position 678 of a PCR product of the MC4R gene, and

c) selecting the animals which have the nucleotide associated with the desired traits in position 678.

29. A method for an indirect selection for a polymorphism in MC4R wherein specific alleles of an alternative DNA marker are used to make the indirect selection wherein the alternative DNA marker is a linked marker near MC4R.

30. The method of claim 29 wherein the linked marker is selected from the group consisting of S0331, BHT0433, and S0313.

31. A method of identifying animals which possess a desired genotype indicative of the metabolic traits of fat content, growth rate, and feed consumption, the method comprising

a) determining an association between a MC4R genotype and a trait of interest by obtaining a sample of animals from a line or breed of interest,

b) preparing genomic DNA from each animal in the sample,

c) determining the genotype of the MC4R gene, and

d) calculating the association between the MC4R genotype and the trait.

32. A method of selecting animals which possess a desired MC4R genotype indicative of the metabolic traits of fat content, growth rate, and feed consumption, the method comprising

a) obtaining a nucleic acid sample from an animal,

b) identifying the genotype of the MC4R gene of the animal, and

c) selecting those animals which have the genotype associated with the desired traits.

FIGURE 1.

CONSENSUS SEQUENCE OF MC4R IN PIG

```
!!NA_SEQUENCE 1.0
con-mc4r.seq  Length: 746  April 9, 1998 16:17  Type: N  Check: 125

  1  ACAAGAATCT GCATTCACCC ATGTACTTTT TCATCTGTAG CCTGGCTGTG
 51  GCTGATATGC TGGTGAGCGT TTCCAATGGG TCAGAAACCA TTGTCATCAC
101  CCTATTAAAC AGCACGGACA CGGACGCACA GAGTTTCACA GTGAATATTG
151  ATAATGTCAT TGACTCAGTG ATCTGTAGCT CCTTACTCGC CTCAATTTGC
201  AGCCTGCTTT CGATTGCAGT GGACAGGTAT TTTACTATCT TTTATGCTCT
251  CCAGTACCAT AACATTATGA CAGTTAAGCG GGTTGGAATC ATCATCAGTT
301  GTATCTGGGC AGTCTGCACG GTGTCGGGTG TTTTGTTCAT CATTTACTCA
351  GATAGCAGTG CTGTTATTAT CTGCCTCATA ACCGTGTTCT TCACCATGCT
401  GGCTCTCATG GCTTCTCTCT ATGTCCACAT GTTCCTCATG GCCAGACTCC
451  ACATTAAGAG GATCGCCGTC CTCCCAGGCA CTGGCACCAT CCGCCAAGGT
501  GCCAACATGA AGGGGGCAAT TACCCTGACC ATCTTGATTG GGTCTTTGT
551  GGTCTGCTGG GCCCCCTTCT TCCTCCACTT AATATTCTAT ATCTCCTGCC
601  CCCAGAATCC ATACTGTGTG TGCTTCATGT CTCACTTTAA TTTGTATCTC
651  ATCCTGATCA TGTGTAATTC CATCATCXAT CCCCTGATTT ATGCACTCCG
701  GAGCCAAGAA CTGAGGAAAA CCTTCAAAGA GATCATCTGT TGCTAT
```

X = G or A, the site of polymorphism.

FIGURE 2.

COMPARISION OF DNA SEQUENCE BETWEEN HUMAN AND PORCINE *MC4R* GENE

```
(Nucleotide) FASTA of: con-mc4r.seq  from: 1 to: 746  April 10, 1998 19:52

TO: s77415.gb_pr  Sequences:        1  Symbols:     1,671  Word Size: 6

Searching with both strands of the query.
Scoring matrix: GenRunData:fastadna.cmp
Constant pamfactor used
Gap creation penalty: 16     Gap extension penalty: 4

The best scores are:                      initl initn   opt..

/usr2/users/rothschi/kwan/mc4r/seq/s77415.gb_pr    Begin: 608  End:  1353
! LOCUS        S77415       1671 bp    ... 3208  3208  3208
/usr2/users/rothschi/kwan/mc4r/seq/s77415.gb_pr    Begin: 388  End:  407
Strand: -
! LOCUS        S77415       1671 bp    ...   70    70    73
\\End of List

con-mc4r.seq
/usr2/users/rothschi/kwan/mc4r/seq/s77415.gb_pr

LOCUS        S77415       1671 bp    DNA             PRI       26-SEP-1995
DEFINITION   melanocortin-4 receptor (human, Genomic, 1671 nt).
ACCESSION    S77415
NID          g998456
KEYWORDS     .
SOURCE       human. . . .

SCORES       Initl:  3208 Initn:  3208 Opt:  3208
  92.2% identity in 746 bp overlap

(SEQ ID NO:3)                                     10        20        30
con-mc4r.seq                                ACAAGAATCTGCATTCACCCATGTACTTTT
                                            ||||||||||||||||||||||||||||||
s77415       ATATCTTAGTGATTGTGGCAATAGCCAAGAACAAGAATCTGCATTCACCCATGTACTTTT
(SEQ ID NO:2) 580       590       600       610       620       630

                       40        50        60        70        80        90
con-mc4r.seq TCATCTGTAGCCTGGCTGTGGCTGATATGCTGGTGAGCGTTTCCAATGGGTCAGAAACCA
             ||||||| ||| ||||:|||||||||||||||||||||||||| ||||| ||||||||||
s77415       TCATCTGCAGCTTGGCTGTGGCTGATATGCTGGTGAGCGTTTCAAATGGATCAGAAACCA
             640       650       660       670       680       690

                      100       110       120       130       140       150
con-mc4r.seq TTGTCATCACCCTATTAAACAGCACGGACACGGACGCACAGAGTTTCACAGTGAATATTG
             || ||||||||||||||||||| || || ||||| |||||||||||||||||||||||||
s77415       TTATCATCACCCTATTAAACAGTACAGATACGGATGCACAGAGTTTCACAGTGAATATTG
             700       710       720       730       740       750
```

FIGURE 2. (con't.)

```
                  160       170       180       190       200       210
con-mc4r.seq ATAATGTCATTGACTCAGTGATCTGTAGCTCCTTACTCGCCTCAATTTGCAGCCTGCTTT
s77415       ATAATGTCATTGACTCGGTGATCTGTAGCTCCTTGCTTGCATCCATTTGCAGCCTGCTTT
             760       770       780       790       800       810

                  220       230       240       250       260       270
con-mc4r.seq CGATTGCAGTGGACAGGTATTTTACTATCTTTTATGCTCTCCAGTACCATAACATTATGA
s77415       CAATTGCAGTGGACAGGTACTTTACTATCTTCTATGCTCTCCAGTACCATAACATTATGA
             820       830       840       850       860       870

                  280       290       300       310       320       330
con-mc4r.seq CAGTTAAGCGGGTTGGAATCATCATCAGTTGTATCTGGGCAGTCTGCACGGTGTCGGGTG
s77415       CAGTTAAGCGGGTTGGGATCAGCATAAGTTGTATCTGGGCAGCTTGCACGGTTTCAGGCA
             880       890       900       910       920       930

                  340       350       360       370       380       390
con-mc4r.seq TTTTGTTCATCATTTACTCAGATAGCAGTGCTGTTATTATCTGCCTCATAACCGTGTTCT
s77415       TTTTGTTCATCATTTACTCAGATAGTAGTGCTGTCATCATCTGCCTCATCACCATGTTCT
             940       950       960       970       980       990

                  400       410       420       430       440       450
con-mc4r.seq TCACCATGCTGGCTCTCATGGCTTCTCTCTATGTCCACATGTTCCTCATGGCCAGACTCC
s77415       TCACCATGCTGGCTCTCATGGCTTCTCTCTATGTCCACATGTTCCTGATGGCCAGGCTTC
            1000      1010      1020      1030      1040      1050

                  460       470       480       490       500       510
con-mc4r.seq ACATTAAGAGGATCGCCGTCCTCCCAGGCACTGGCACCATCCGCCAAGGTGCCAACATGA
s77415       ACATTAAGAGGATTGCTGTCCTCCCCGGCACTGGTGCCATCCGCCAAGGTGCCAATATGA
            1060      1070      1080      1090      1100      1110

                  520       530       540       550       560       570
con-mc4r.seq AGGGGGCAATTACCCTGACCATCTTGATTGGGGTCTTTGTGGTCTGCTGGGCCCCCTTCT
s77415       AGGGAGCGATTACCTTGACCATCCTGATTGGCGTCTTTGTTGTCTGCTGGGCCCCATTCT
            1120      1130      1140      1150      1160      1170

                  580       590       600       610       620       630
con-mc4r.seq TCCTCCACTTAATATTCTATATCTCCTGCCCCCAGAATCCATACTGTGTGTGCTTCATGT
s77415       TCCTCCACTTAATATTCTACATCTCTTGTCCTCAGAATCCATATTGTGTGTGCTTCATGT
            1180      1190      1200      1210      1220      1230

                  640       650       660       670       680       690
con-mc4r.seq CTCACTTTAATTTGTATCTCATCCTGATCATGTGTAATTCCATCATCAATCCCCTGATTT
s77415       CTCACTTTAACTTGTATCTCATACTGATCATGTGTAATTCAATCATCGATCCTCTGATTT
            1240      1250      1260      1270      1280      1290

                  700       710       720       730       740
con-mc4r.seq ATGCACTCCGGAGCCAAGAACTGAGGAAAACCTTCAAAGAGATCATCTGTTGCTAT
s77415       ATGCACTCCGGAGTCAAGAACTGAGGAAAACCTTCAAAGAGATCATCTGTTGCTATCCCC
            1300      1310      1320      1330      1340      1350

s77415       TGGGAGGCCTTTGTGACTTGTCTAGCAGATATTAAATGGGGACAGAGCACGCAATATAGG
            1360      1370      1380      1390      1400      1410
```

FIGURE 3.

COMPARISON OF AMINO ACID SEQUENCE BETWEEN HUMAN AND PORCINE *MC4R* GENE

```
(Peptide) TFASTA of: human.pep  from: 1 to: 332  April 9, 1998 16:18

REFORMAT of: human  check: 9754  from: 1  to: 332  March 7, 1998 18:43
(No documentation)

TO: mc4r-alle*.seq  Sequences:        2  Symbols:      1,492  Word Size: 2

Searching all six frames.
Scoring matrix: GenRunData:blosum50.cmp
Variable pamfactor used
Gap creation penalty: 16      Gap extension penalty: 4


The best scores are:                       frame initl initn   opt..

/usr2/users/rothschi/kwan/mc4r/seq/mc4r-allel.seq    Begin: 3  End:  746
! mc4r-allel.seq  Length: 746  April ...(3)  1602  1602  1602
/usr2/users/rothschi/kwan/mc4r/seq/mc4r-alle2.seq    Begin: 3  End:  746
! mc4r-alle2.seq  Length: 746  April ...(3)  1596  1596  1596


SCORES  Frame: (3) Initl:  1602 Initn:  1602 Opt:  1602
  97.6% identity in 248 aa overlap

(SEQ ID NO:4)     50        60        70        80        90       100
 human.pep   QLFVSPEVFVTLGVISLLENILVIVAIAKNKNLHSPMYFFICSLAVADMLVSVSNGSETI
                                           ||||||||||||||||||||||||||||||
 mc4r-allel                                KNLHSPMYFFICSLAVADMLVSVSNGSETI
(SEQ ID NO:5)                                      10        20        30

                 110       120       130       140       150       160
 human.pep   IITLLNSTDTDAQSFTVNIDNVIDSVICSSLLASICSLLSIAVDRYFTIFYALQYHNIMT
             :|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 mc4r-allel  VITLLNSTDTDAQSFTVNIDNVIDSVICSSLLASICSLLSIAVDRYFTIFYALQYHNIMT
                     40        50        60        70        80        90

                 170       180       190       200       210       220
 human.pep   VKRVGISISCIWAACTVSGILFIIYSDSSAVIICLITMFFTMLALMASLYVHMFLMARLH
             |||||| ||||||:|||||:|||||||||||||||||:||||||||||||||||||||||
 mc4r-allel  VKRVGIIISCIWAVCTVSGVLFIIYSDSSAVIICLITVFFTMLALMASLYVHMFLMARLH
                    100       110       120       130       140       150

                 230       240       250       260       270       280
 human.pep   IKRIAVLPGTGAIRQGANMKGAITLTILIGVFVVCWAPFFLHLIFYISCPQNPYCVCFMS
             |||||||||||:||||||||||||||||||||||||||||||||||||||||||||||||
 mc4r-allel  IKRIAVLPGTGTIRQGANMKGAITLTILIGVFVVCWAPFFLHLIFYISCPQNPYCVCFMS
                    160       170       180       190       200       210

                 290       300       310       320       330
 human.pep   HFNLYLILIMCNSIIDPLIYALRSQELRKTFKEIICCYPLGGLCDLSSRY
             ||||||||||||||||||||||||||||||||||||||
 mc4r-allel  HFNLYLILIMCNSIIDPLIYALRSQELRKTFKEIICCY
                    220       230       240
```

FIGURE 3. (cont.)

```
human.pep
/usr2/users/rothschi/kwan/mc4r/seq/mc4r-alle2.seq


SCORES   Frame: (3) Initl:   1596 Initn:   1596 Opt:   1596
  97.2% identity in 248 aa overlap

                   50        60        70        80        90       100
human.pep   QLFVSPEVFVTLGVISLLENILVIVAIAKNKNLHSPMYFFICSLAVADMLVSVSNGSETI
                                            ||||||||||||||||||||||||||||||
mc4r-alle2                                  KNLHSPMYFFICSLAVADMLVSVSNGSETI
                                                    10        20        30

                  110       120       130       140       150       160
human.pep   IITLLNSTDTDAQSFTVNIDNVIDSVICSSLLASICSLLSIAVDRYFTIFYALQYHNIMT
            :|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
mc4r-alle2  VITLLNSTDTDAQSFTVNIDNVIDSVICSSLLASICSLLSIAVDRYFTIFYALQYHNIMT
                    40        50        60        70        80        90

                  170       180       190       200       210       220
human.pep   VKRVGISISCIWAACTVSGILFIIYSDSSAVIICLITMFFTMLALMASLYVHMFLMARLH
            |||||| |||||:|||||:|||||||||||||||||:|||||||||||||||||||||||
mc4r-alle2  VKRVGIIISCIWAVCTVSGVLFIIYSDSSAVIICLITVFFTMLALMASLYVHMFLMARLH
                   100       110       120       130       140       150

                  230       240       250       260       270       280
human.pep   IKRIAVLPGTGAIRQGANMKGAITLTILIGVFVVCWAPFFLHLIFYISCPQNPYCVCFMS
            ||||||||||:|||||||||||||||||||||||||||||||||||||||||||||||||
mc4r-alle2  IKRIAVLPGTGTIRQGANMKGAITLTILIGVFVVCWAPFFLHLIFYISCPQNPYCVCFMS
                   160       170       180       190       200       210

                  290       300       310       320       330
human.pep   HFNLYLILIMCNSIIDPLIYALRSQELRKTFKEIICCYPLGGLCDLSSRY
            |||||||||||||||:||||||||||||||||||||||
mc4r-alle2  HFNLYLILIMCNSIINPLIYALRSQELRKTFKEIICCY
                   220       230       240


! CPU time used:
!         Database scan:   0:00:00.2
! Post-scan processing:   0:00:00.1
!        Total CPU time:   0:00:00.4
! Output File: human.tfasta
```

FIGURE 4a.

LINKAGE REPORT OF TWO-POINT ANALYSIS

New locus MC4R
in sex-equal linkage analyses with existing loci

S0082	MC4R	rec. fracs.=	0.05,	lods =	14.74
CGA	MC4R	rec. fracs.=	0.14,	lods =	6.88
S0020	MC4R	rec. fracs.=	0.18,	lods =	5.32
S0079	MC4R	rec. fracs.=	0.12,	lods =	10.35
S0155	MC4R	rec. fracs.=	0.14,	lods =	7.68
S0122	MC4R	rec. fracs.=	0.18,	lods =	5.17
S0313	MC4R	rec. fracs.=	0.00,	lods =	17.76
S0312	MC4R	rec. fracs.=	0.20,	lods =	5.60
S0311	MC4R	rec. fracs.=	0.17,	lods =	7.18
S0416	MC4R	rec. fracs.=	0.20,	lods =	3.21
S0331	MC4R	rec. fracs.=	0.02,	lods =	21.91
S0396	MC4R	rec. fracs.=	0.16,	lods =	7.85
BHT0433	MC4R	rec. fracs.=	0.02,	lods =	21.32
S0536	MC4R	rec. fracs.=	0.03,	lods =	15.61
CAPN3	MC4R	rec. fracs.=	0.12,	lods =	6.65
KGF	MC4R	rec. fracs.=	0.09,	lods =	6.46
MEF2A	MC4R	rec. fracs.=	0.05,	lods =	14.36
MC4R	MC4R	rec. fracs.=	0.00,	lods =	26.19

FIGURE 4b.

new locus MC4R

in sex-specific (female/male) linkage analyses with existing loci

S0082	MC4R	rec. fracs.=	0.00	0.09,	lods	=	15.86
CGA	MC4R	rec. fracs.=	0.07	0.22,	lods	=	7.46
S0020	MC4R	rec. fracs.=	0.00	0.25,	lods	=	6.33
S0079	MC4R	rec. fracs.=	0.00	0.19,	lods	=	11.48
S0155	MC4R	rec. fracs.=	0.00	0.24,	lods	=	9.98
S0122	MC4R	rec. fracs.=	0.00	0.27,	lods	=	7.10
S0313	MC4R	rec. fracs.=	0.00	0.00,	lods	=	17.76
S0312	MC4R	rec. fracs.=	0.04	0.29,	lods	=	7.45
S0311	MC4R	rec. fracs.=	0.00	0.28,	lods	=	9.02
S0416	MC4R	rec. fracs.=	0.00	0.31,	lods	=	4.17
S0331	MC4R	rec. fracs.=	0.05	0.00,	lods	=	22.14
S0396	MC4R	rec. fracs.=	0.03	0.24,	lods	=	9.33
BHT0385	MC4R	rec. fracs.=	0.14	0.36,	lods	=	3.46
BHT0433	MC4R	rec. fracs.=	0.05	0.00,	lods	=	21.82
S0536	MC4R	rec. fracs.=	0.00	0.05,	lods	=	15.77
CAPN3	MC4R	rec. fracs.=	0.00	0.18,	lods	=	7.35
KGF	MC4R	rec. fracs.=	0.00	0.17,	lods	=	6.74
MEF2A	MC4R	rec. fracs.=	0.10	0.00,	lods	=	14.52
MC4R	MC4R	rec. fracs.=	0.00	0.00,	lods	=	26.19

FIGURE 4c.

LINKAGE REPORT OF MULTIPOINT ANALYSIS

Sex_averaged map (recomb. frac., Kosambi cM):

0	ESR			0.0
		0.18	18.4	
1	S0008			18.4
		0.12	11.9	
7	CGA			30.3
		0.03	2.8	
3	S0312			33.1
		0.05	4.9	
4	S0122			38.1
		0.09	9.4	
8	KGF			47.4
		0.06	5.8	
6	CAPN3			53.2
		0.02	2.5	
9	MEF2A			55.7
		0.06	6.1	
5	MC4R			61.8
		0.06	5.6	
10	S0313			67.4
		0.00	0.0	
11	S0082			67.4
		0.03	3.4	
12	S0079			70.8
		0.03	2.5	
14	S0142			73.3
		0.01	1.0	
13	S0020			74.4
		0.04	4.3	
15	S0311			78.7
		0.00	0.0	
16	S0155			78.7
		0.12	12.2	
17	S0113			90.9
		0.20	21.0	
18	S0302			111.9
		0.22	23.4	
19	S0112			135.3

* denotes recomb. frac. held fixed in this analysis. log10_like = -305.098

FIGURE 5.

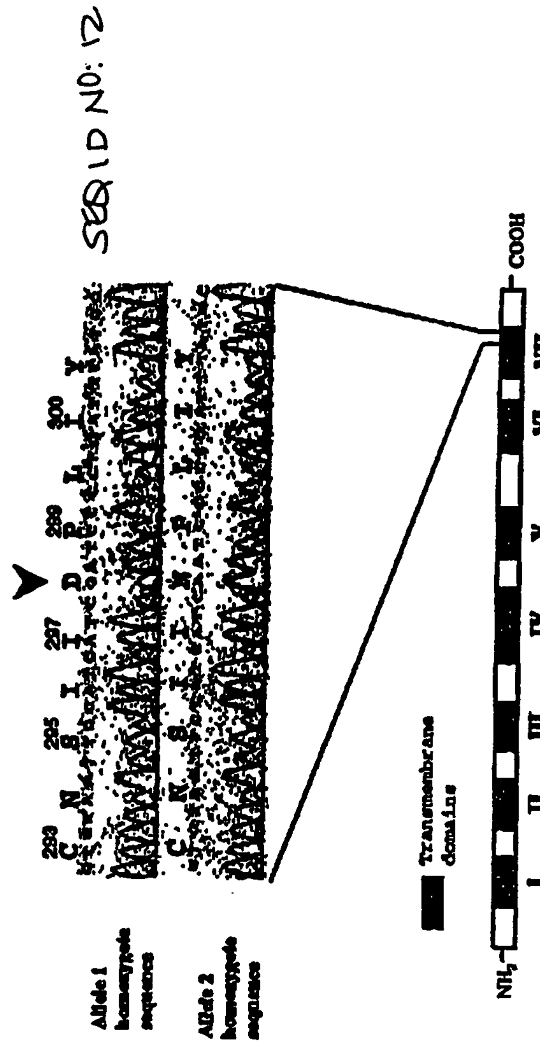

SEQ ID NO: 12
Allele 1 homozygote sequence
Allele 2 homozygote sequence
Transmembrane domains
NH2
COOH
I
II
III
IV
V
VI
VII

FIGURE 6.

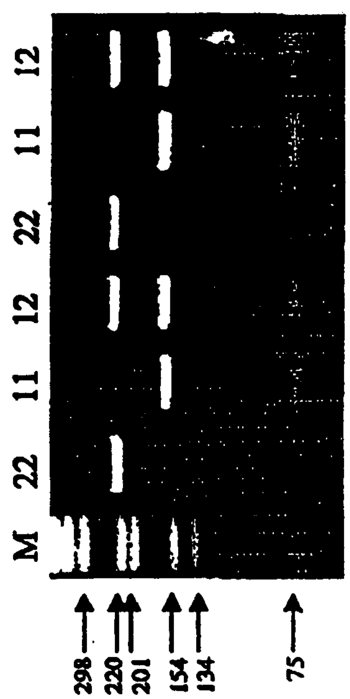
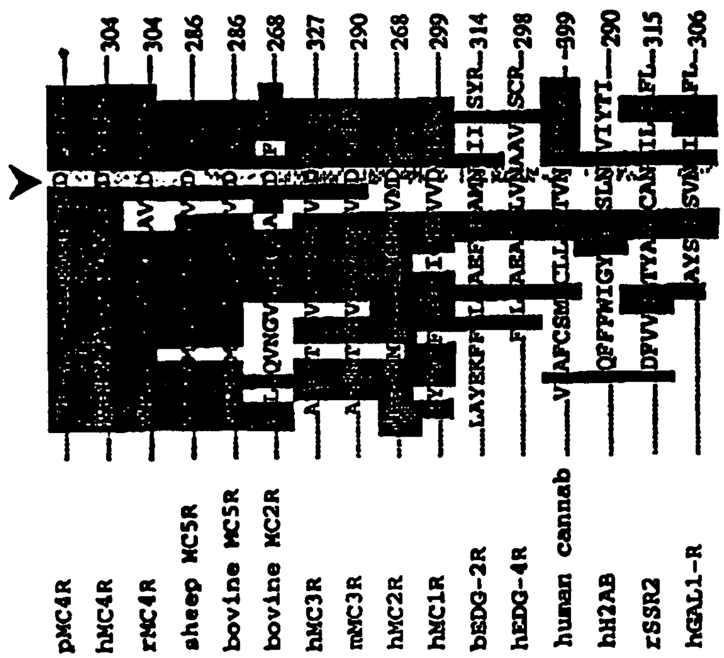


FIGURE 7.


pMC4R
hMC4R 304
rMC4R 304
sheep MC5R 286
bovine MC5R 286
bovine MC2R 268
hMC3R 327
mMC3R 290
hMC2R 268
hMC1R 299
bEDG-2R 314
hEDG-4R 298
human cannab 399
hH2AB 290
rSSR2 315
hGAL1-R 306

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(54) Title: MELANOCORTIN-4 RECEPTOR GENE AND USE AS A GENETIC MARKER FOR FAT CONTENT, WEIGHT GAIN, AND/OR FEED CONSUMPTION OF ANIMALS

(57) Abstract

Genetic markers in the porcine melanocortin-4 receptor (MC4R) gene are disclosed which are associated with fat content, growth rate, and feed consumption. Further, novel sequence data from regions of the gene are disclosed which may be used in a PCR test to screen for the presence of the marker. The genetic marker may be used to screen animals for breeding purposes which have the desired traits regarding fat content, growth rate, and feed consumption. Kits which take advantage of the PCR test are also disclosed.

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Inte. nal Application No
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A. CLASSIFICATION OF SUBJECT MATTER
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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GOTODA T ET AL: "Molecular screening of the human melanocortin-4 receptor gene: identification of a missense variant showing no association with obesity, plasma glucose, or insulin" DIABETOLOGIA, vol. 40, no. 8, August 1997 (1997-08), pages 976-79, XP000866534 ---	
A	ANDERSSON L ET AL: "GENETIC MAPPING OF QUANTITATIVE TRAIT LOCI FOR GROWTH AND FATNESS IN PIGS" SCIENCE,US,AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE,, vol. 263, page 1771-1774 XP002018359 ISSN: 0036-8075 --- -/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
21 January 2000	01/02/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Osborne, H

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/16862

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97 23644 A (COFOK B V ;BEHEERMAATSCHAPPIJ VARKENSVERB (NL); INSTITUUT VOOR DIE) 3 July 1997 (1997-07-03)	
A	WO 97 47316 A (MILLENNIUM PHARMACEUTICALS INC) 18 December 1997 (1997-12-18)	

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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 99/16862

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
WO 9723644	A	03-07-1997	AU	1212797	A	17-07-1997
			CA	2239812	A	03-07-1997
			EP	0868533	A	07-10-1998
			US	5940198	A	17-08-1999
WO 9747316	A	18-12-1997	US	5908609	A	01-06-1999
			US	5932779	A	03-08-1999
			AU	3383697	A	07-01-1998
			CA	2257857	A	18-12-1997
			CN	1227496	A	01-09-1999
			EP	0915706	A	19-05-1999